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Long Term Response to Interferon Treatment in Chronic Hepatitis C Patients Is Associated With a Significant Reduction in Anti-E1 Envelope Antibody Titers

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Interferon (IFN) alfa has been used widely for the treatment of chronic hepatitis C virus (HCV) infections but only a small number of patients treated have shown a sustained biochemical and virological response. Anti-envelope E1 and E2 antibody titers were assessed retrospectively before, during, and after treatment with IFN in order to evaluate their usefulness for the prediction and monitoring of therapy outcome in 115 patients infected chronically with HCV genotype 1b. At baseline, E2 induced more frequent and stronger immunogenic responses than E1, irrespective of patient response to therapy. E1 and E2 antibodies also tended to be higher in patients with a long-term or a transient response to IFN treatment than in patients who were absolute non-responders. In most patients, E1 and E2 antibody levels tended to be lower after treatment. This reduction was most pronounced and occurred most frequently in long-term responders to therapy. In this patient group, the reduction of E1 antibodies was more pronounced than that of E2 antibodies. In contrast to E2 antibodies, the decrease of E1 antibodies could already be observed at the end of therapy (week 24) and was significantly larger ($p<0.05$) than that observed in relapsers and non-responders. Thus, a sustained elevation of E1 antibodies seems to be associated with ongoing infection even when HCV RNA levels were undetectable in serum. Monitoring of E1 antibody titers may represent a useful additional marker to discriminate sustained responders from those who relapse in patients receiving interferon therapy. ***J. Med. Virol. 60:126–132, 2000.***

KEY WORDS: hepatitis C virus; interferon therapy; humoral response; anti-envelope antibodies; prediction of therapy outcome; monitoring outcome of therapy

INTRODUCTION

Hepatitis C virus (HCV) is the main cause of non-A, non-B hepatitis [Choo et al., 1989]. Its genome is a single-stranded linear RNA molecule of positive sense composed of a structural region (core and envelope glycoproteins E1 and E2) and a non-structural region consisting of six parts (NS2, NS3, NS4A/B and NS5A/B) [Grakoui et al., 1993; Hijikata et al., 1993]. The virus is not cleared easily by the host's immunological defense mechanisms and, thus, a persistent infection develops in over 85% of patients leading to chronic hepatitis, cirrhosis, and hepatocellular carcinoma [Alter et al., 1989; Barrera et al, 1995; Fattovich et al., 1997]. Interferon (IFN) alfa remains the only drug that is effective in the treatment of chronic hepatitis C. A 6 month course of treatment with 3 million units of IFN three times a week for 24 weeks was used in numerous studies and resulted in a sustained biochemical and virological response in only 10 to 20% of treated patients [Hoofnagle and di Bisceglie, 1997; Lindsay, 1997]. Ribavirin, a nucleoside analogue, has been evaluated as a treatment for chronic hepatitis C alone and in combination with IFN alfa. With ribavirin alone, almost all patients remain viraemic. Combination

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therapy however has been demonstrated to significantly improve the sustained biochemical and virological response when compared to IFN alone [Reichard et al., 1997].

As only a minority of patients with chronic hepatitis C benefits from treatment with IFN, it would be interesting to develop an algorithm that predicts therapy outcome on an individual basis in order to reduce medical expenses and to spare patients from unnecessary treatment. Recently, pretreatment prognostic features that have been associated with a favourable response to IFN therapy include absence of cirrhosis [Shindo et al., 1997] , low levels of HCV RNA [Ballardini et al., 1997] , and the presence of viral genotype other than 1[Martinot Peignoux et al, 1995; Shiratori et al., 1997]. Recently, data were published on the relationship between therapy outcome and humoral response towards HCV-related proteins. A long term response to IFN seems to be correlated with a high baseline NS5A reactivity [Frangeul et al., 1998] or with a decline of anticore antibodies during treatment [Hirayama et al., 1997]. An earlier study, using recombinant envelope proteins produced in yeast, showed that a long term response correlated with the appearance of E1 antibodies and the disappearance of E2 antibodies [Saracco et al, 1994].

The envelope proteins constitute the outer surface of the virion embedded in a host-derived membrane. Antibodies directed towards the outer surface envelope epitopes are thus the prime candidate for virus neutralization and/or clearance and may play an important role during IFN treatment. The detection of anti-envelope antibodies in sera from chronic HCV patients varies depending on the recombinant capturing protein and the assay procedure used. Optimal antibody detection is achieved using an enzyme linked immunoassay with the capture antigen being a native, glycosylated protein synthesized, processed and secreted by a mammalian cell expression system [Chien et al, 1993; Fournillier Jacob et al., 1996; Harada et al., 1995; Hussy et al., 1997; Inudoh et al., 1996; Lee et al., 1997; Lesniewski et al., 1995; Maertens and Stuyver, 1997; Saracco et al., 1994; Yan et al., 1994; Yuki et al., 1996; Zaaijer et al., 1994]. Anti-envelope antibodies may also be type-specific [Maertens and Stuyver, 1997]. An important characteristic of the HCV genome is its sequence heterogeneity [Bukh et al., 1995]. Individual isolates consist of closely related, yet heterogeneous populations of viral genomes (quasispecies). Comparing the genomic nucleotide sequences from different isolates enables classification of the viruses into several genotypes and subtypes. The separate HCV genome regions show different degrees of heterogeneity dictated by functional constraints and immune pressure. The core region is most conserved while both envelope proteins exhibit higher variability than the average diversity of the whole genome.

In this study, the usefulness of E1 and E2 antibody assessment was examined for predicting and monitoring of IFN therapy outcome in chronic hepatitis C genotype 1b patients. Antibodies were detected using recombinant, mammalian expressed proteins with a HCV 1b sequence as the capturing antigens.

MATERIALS AND METHODS

Patients

The patients included in this study were selected from a larger cohort of patients suffering from chronic hepatitis C that were enrolled in a Benelux multicenter study [Brouwer et al., 1998] that compared the efficacy of standard treatment with IFN alfa with that of an experimental IFN regimen. The standard treatment consisted of subcutaneous injections of IFN alfa 2b (Intron A, Schering Plough Corporation, Kenilworth, NJ) at 3 million units (MU) three times a week for 24 weeks, while the experimental regimen consisted of the administration of 6, 3 and 1 MU thrice weekly for 8, 4, and 12 weeks, respectively. After cessation of IFN treatment, the patients were followed further for an additional 52 weeks. Serum alanine aminotransferase (ALT) levels and HCV RNA were examined before, during, or after IFN therapy. The latter test was carried out using a qualitative, standardized polymerase chain reaction (PCR) assay that had been validated with the EUROHEP proficiency panel. All patients had elevated serum ALT levels on at least three occasions with monthly intervals and showed HCV RNA before treatment. Patients were classified as sustained responders when normalization of ALT levels and absence of HCV RNA occurred for at least 6 months after cessation of therapy. Normalization of ALT was considered to have occurred when the serum ALT levels did not exceed the upper limit of normal on at least two successive occasions with at least a 4-week interval between tests. Relapsers showed only a transient normalization of ALT and/or disappearance of HCV RNA. Non-responders to therapy had persistently elevated ALT levels and/or detectable HCV RNA throughout therapy and follow-up.

Out of the total cohort of treated patients, 115 individuals infected with HCV genotype 1b were selected from whom sufficient serum was still available. HCV genotypes were assessed at baseline with the HCV-specific line probe assay (INNO-LIPA HCV II, Innogenetics, Ghent, Belgium). Before therapy, plasma HCV RNA concentrations were quantified by branched DNA (Quantiplex version 1.0, Chiron Corporation, Emeryville, CA). The results are expressed as multiples of 10^5 genome equivalents per milliliter (cut-off: 2×10^5eq/ml). Patients with HCV RNA concentrations below the detection limit of the branched DNA assay were given an arbitrary number of 1×10^5 genome equivalents per ml for use in statistical analysis.

HCV E1 and E2 Antibody Detection

Anti-HCV envelope1 (E1) and envelope2 (E2) immunoglobulin levels before (week 0), upon termination (week 24) and after (weeks 36 to 78) IFN therapy were measured using the Innotest HCV E1 antibody and Innotest HCV E2 antibody prototype versions (Innoge-

TABLE I. Baseline Prevalence and Titer of E1 and E2 Antibodies in HCV 1b Patients According to IFN Therapy Outcome

		E1 antibodies					E2 antibodies		
		Positive patients (%)	Median (IQR)[a]				Positive patients (%)	Median (IQR)	
Sustained responders	n = 13	8 (61)	7.04 (3.98–9.53)		Sustained responders	n = 13	12 (92)	9.76 (5.55–15.3)	
Relapsers	n = 36	27 (75)	5.79 (2.74–8.60)	*	Relapsers	n = 29	26 (89)	10.20 (7.14–13.6)	
Non-responders	n = 66	37 (56)	3.68 (2.26–6.54)		Non-responders	n = 60	45 (75)	6.32 (3.54–11.25)	*
Total	n = 115	72 (63)			Total	n = 102	83 (81)		

[a]IQR, interquartile range
*$P < 0.05$.

netics). The recombinant E1 and E2 proteins employed as the capturing antigens in these indirect solid-phase enzyme-immunoassays were both high quality, glycosylated proteins expressed from vaccinia constructs in mammalian cells and purified to homogeneity of >99.7% [Bosman et al., 1997]. The E1 and E2 cDNAs were cloned from a European subtype 1b HCV isolate. These encode proteins spanning the E1 (aa 192 to 326) and E2 (aa 384 to 673) ectodomains in their mature form. Serum samples were diluted 1/20 in the assay. E1- and E2-specific antibodies present in the serum will bind to the capturing proteins. These antibodies are then detected by a horseradish peroxidase-labeled polyclonal rabbit anti-human IgG. After addition of tetramethylbenzidine substrate solution, positive samples develop a blue color. The reaction is stopped by addition of sulfuric acid yielding a yellow color that is measured at 450 nm. Serum from uninfected healthy donors and from HCV-infected, anti-E1 and/or -E2 positive patients were included as negative and positive controls, respectively. A cut-off was calculated using the optical density values of negative and positive controls. Signal to cut-off ratios of >1.5 were considered positive.

Statistical Analysis

The significance of differences observed between the different patient groups were analyzed using the Mann-Whitney U test.

RESULTS

Prevalence of Baseline E1 and E2 Antibodies and Its Correlation With Response to IFN Therapy

Baseline E1 and E2 antibody levels were examined in 115 chronic HCV 1b patients (male/female ratio: 60/55; mean age: 49 years) of whom 66 (57%) showed no response to IFN therapy (non-responders), 36 (31%) were relapsers, and 13 (11%) were sustained responders. Baseline E2 antibody levels could not be measured in 7 relapsers and 6 non-responders because serum was no longer available (Table I).

Table I shows that 63% of patients displayed an anti-E1 response whereas 81% were positive for anti-E2. Anti-E2 responses were not only more frequent but also higher in titer as compared to anti-E1 responses, irrespective of therapy outcome. E1 and E2 antibody levels tended to be higher in sustained responders and relapsers than in non-responders. However, these pretreatment E1 and E2 antibody levels did not allow for prediction of the therapeutic outcome of the individual patient.

TABLE II. Baseline HCV Viraemic Titer in HCV 1b Patients According to IFN Therapy Outcome

		HCV-RNA[a]	
Sustained responders	n = 13	1.0 (1.0–9.7)	*
Relapsers	n = 36	14.0 (7.1–50.5)	*
Non-responders	n = 66	27.1 (7.7–67.1)	

[a]Median (50%) genome equivalents × 10^5/ml interquartile ranges (25%–75%) between brackets.
*$P < 0.005$.

Although baseline E1 and E2 antibody concentrations did not correlate with pretreatment serum HCV RNA titers or ALT levels (data not shown), a significant correlation was observed between pretreatment viraemic levels and response to therapy: low viral loads were found in sera from sustained responders and higher viral loads were observed in sera from relapsers and non-responders (Table II). Because of the diametrically opposite levels of HCV RNA and envelope antibodies at baseline, we calculated the ratios between pretreatment E1 or E2 antibodies and HCV RNA titers for each patient. Since anti-envelope antibody titers tended to be higher and HCV RNA titers lower in SR as compared to NR and REL, these anti-envelope/HCV RNA ratios turned out to be clear discriminators between the different patient groups (Fig. 1). The observed differences in the anti-E1/HCV RNA and anti-E2/HCV RNA ratio between non-responders, relapsers and sustained responders were significant at the $P<0.05$ level.

To examine whether these anti-E1/HCV RNA or anti-E2/HCV RNA ratios could be useful for the selection of patients that would be sustained responders to IFN therapy, an anti-E1/HCV RNA ratio of 27.1 (the 75% range of non-responders) as the cut-off level beyond which a subject would be considered a sustained responder was chosen (Table III). If this value is used as the decision limit, medical costs and treatment mor-

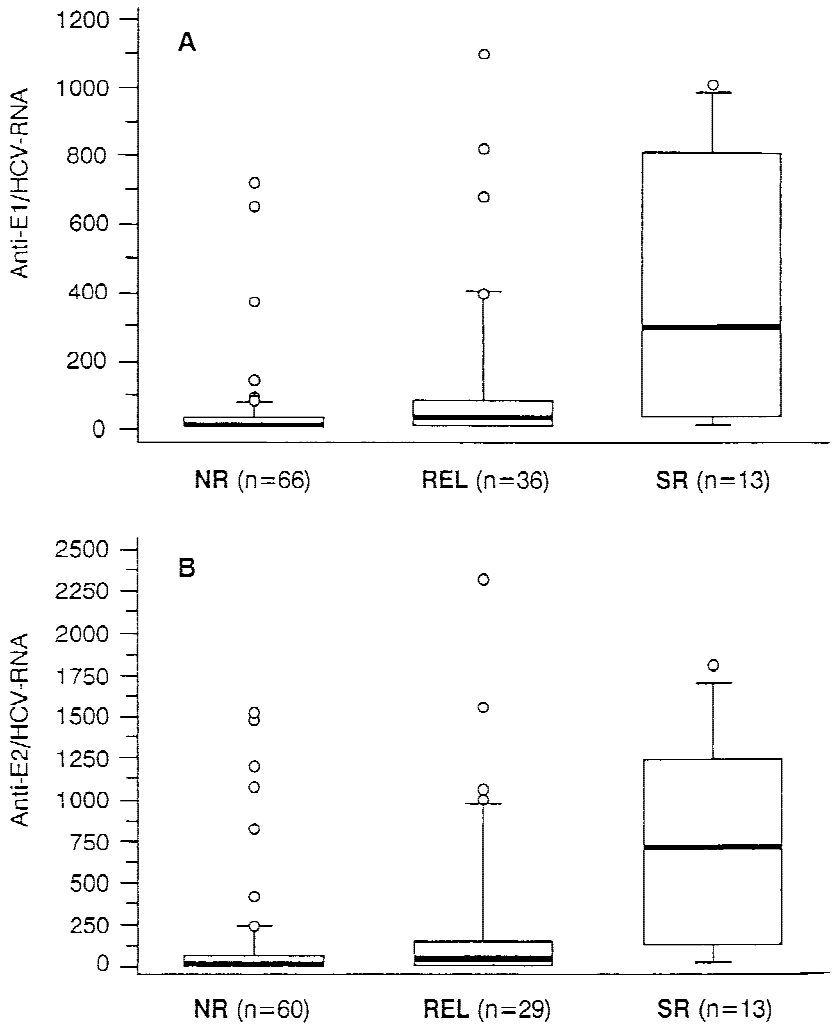

Fig. 1. Box plot of anti-envelope antibody/HCV RNA ratios in non-responders (NR), relapsers (REL), and sustained responders (SR) to IFN therapy. The baseline anti-E1/HCV RNA **(A)** and anti-E2/HCV RNA **(B)** ratios were calculated for each patient. Respective data were grouped according to therapy outcome. Median (50%) ratio values and interquartile ranges (25%) are indicated by the heavy line and boxes, respectively. Flags represent 10 to 90% ranges.

TABLE III. Selection of HCV 1b Patients for IFN Therapy on Basis of the Individual Baseline Anti-E1/HCV-RNA Ratio or HCV-RNA Titer

	Number of patients treated with interferon	Number of patients with (E1/HCV-RNA) × 100 exceeding 27.1 (75% range of NR) (% of patients)	Number of patients with a HCV titer below 9.7 × 105 eq/ml (75% range of SR) (% of patients)
Sustained responders	13	10 (77)	10 (77)
Relapsers	36	17 (47)	13 (36)
Non-responders	66	15 (23)	22 (33)
Total	115	42 (37)	45 (39)

bidity would have been lowered because 73 patients (63%) would not have received IFN therapy and the sustained response rate among those who were offered treatment would have been increased from 11% (13/115) to 24% (10/42). Unfortunately, this selection strategy would also have withheld therapy to 3 (23%) patients who would have achieved a sustained response.

The sensitivity, specificity, and positive predictive value of the anti-E1/HCV RNA ratio and of the absolute HCV RNA titers in the prediction of response to IFN therapy were also compared. The HCV RNA titer below which a subject was considered a sustained responder was taken at 9.7×10^5 genome equivalents per ml (the 75% range of SR) (Table III). This serum HCV RNA level was quite close to the internationally accepted decision limit of 1×10^6 eq/ml (Davis and Lau, 1997). At these decision limits for anti-E1/HCV RNA ratios and HCV RNA, the diagnostic sensitivities (the

TABLE IV. Evolution of E1 and E2 Antibody Concentrations: Comparison of Values Obtained Before and After IFN Treatment

		Antibody levels		Evolution of antibody levels	
Anti-E1		Pre-IFN values[a]	Post-IFN values[a]	(Pre-post) values[a]	% patients with >15% reduction
Non-responders	(n = 32)	3.78	3.29	0.48	44
Relapsers	(n = 25)	5.45 **	4.60	0.35 ** *	36
Sustained responders	(n = 8)	7.04	3.77	2.5	100
Anti-E2		Pre-IFN values[a]	Post-IFN values[a]	(Pre-post) values[a]	% patients with >15 % reduction
Non-responders	(n = 38)	5.96 **	6.37 **	0.39	42
Relapsers	(n = 24)	10.25	9.81	0.28 *	33
Sustained responders	(n = 12)	9.76	8.56	1.43	75

[a]median value
*$P < 0.01$
**$P < 0.05$.

proportion of responders who have the factor) for both tests were equal and reached 77% (10/13). The diagnostic specificities (the proportion of non-sustained responders (i.e. relapsers and non-responders) without the factor) of both tests were 68% (70/102) and 65% (67/102) for the anti-E1/HCV RNA ratio and HCV RNA titer, respectively. The positive predictive values (the proportion of patients with the factor who respond to treatment) of both tests were 23 % (10/42) and 22 % (10/45) for anti-E1/HCV RNA ratio and HCV RNA titers, respectively. One major difference between the anti-E1/HCV RNA ratio and HCV RNA was that almost one third less non-responders were treated with IFN when the ratio rather than the HCV RNA level would have been chosen as the decision factor. Very similar data were obtained when the anti-E2/HCV RNA ratio was employed for the calculations described above (data not shown).

Evolution of E1 and E2 Antibodies During and After IFN Therapy and Its Correlation With Therapy Outcome

Post-treatment E1 and E2 antibodies were quantified only in patients that were positive for E1 and/or E2 antibodies at baseline. Sera were collected between 12 and 52 weeks after the cessation of therapy (Table IV). In most chronic HCV 1b patients treated with IFN, the E1 and E2 antibody levels tended to be lower after therapy. The decline of both E1 and E2 antibodies was most pronounced in sustained responders. Indeed all sustained responders showed a decline in E1 antibodies of at least 15% whereas only 44% and 36% of non-responders and relapsers, respectively, showed such a decline. A similar pattern was seen for the E2 levels. Finally, in sustained responders the reduction of E1 antibodies was larger than that of E2 antibodies.

The decline in E1 antibodies could also be observed at an earlier timepoint. Already after 12 to 36 weeks following the start of IFN therapy, E1 levels tended to be lower than at the start of therapy in sustained responders. This was not so in non-responders or in relapsers (Table V). The difference in decline of E1 antibodies between sustained responders and relapsers was significant at the $P<0.05$ level. At this timepoint, all patients with a sustained response showed a reduction in E1 antibody levels of more than 15%. This occurred in only half of the non-responders and relapsers. A significant drop in E1 or E2 antibody titers could however not yet be observed at 4 weeks after the start of therapy (data not shown).

TABLE V. Evolution of E1 Antibody Concentrations: Comparison of Values Obtained Before IFN Treatment and at the End of Therapy (Week 24)

		Evolution of antibody levels	
Anti-E1		(Pre-end) values #	% patients with >15% reduction
Non-responders	n = 27	0.44	52
Relapsers	n = 22	0.49 * *	41
Sustained responders	n = 4	2.30	100

#, median value; *$P < 0.05$.

DISCUSSION

In this study, the prevalence of antibodies recognizing HCV envelope proteins E1 and E2 was examined in sera of chronic hepatitis patients infected with HCV genotype 1b. The envelope regions exhibit the highest variability within the whole HCV RNA genome [Stuyver et al., 1993; Stuyver et al., 1994]. Therefore, recombinant envelope proteins with a HCV 1b sequence were used in the enzyme immunoassays to maximize the match between the infecting virus and the capturing antigens. Over 80% of all patients had high titers of antibodies to E2. A similar high prevalence of E2 antibodies in chronic HCV-infected individuals was reported by other investigators using mammalian cell-derived recombinant proteins as capturing antigens [Chien et al., 1993; Saracco et al., 1994; Zaaijer et al., 1994; Harada et al., 1995; Lesniewski et al., 1995; Fournillier Jacob et al., 1996; Inudoh et al., 1996; Lee et al., 1996; Yuki et al, 1996; Lee et al., 1997]. E2 antibodies are also thought to exert broad genotype cross-reactivity by recognition of more conserved epi-

topes [Mink et al., 1994; Yuki et al., 1996; Psichogiou et al., 1997]. Thus, due to the strong and broad immunogenicity of the protein, inclusion of an E2 protein in future anti-HCV serodiagnostic kits may still improve sensitivity and specificity of currently available third generation immunoassays.

Over 60% of all patients had reactivity to E1. E1 antibodies are mostly type-specific [Maertens and Stuyver, 1997] and the high prevalence may thus result from matching the genotype of the *in vivo* infecting virus with that of the capturing antigen. To our knowledge, only one paper has been published on E1 antibody detection in chronic HCV patients using recombinant proteins, produced by a mammalian expression system [Kohara et al., 1992]. The detection rate was low (14%) probably due to denaturing purification technique or inferior detection methods used.

In an effort to determine whether patients could be preselected for treatment, several studies have attempted to identify clinical or virological features that may distinguish chronic hepatitis C patients who may or may not respond to interferon therapy [Serfaty et al., 1994; Davis and Lau, 1997]. Pretreatment prognostic features that were associated with a favorable response to short courses (6 months) of IFN treatment included absence of cirrhosis [Shindo et al., 1997], low levels of HCV RNA in the serum or liver [Ballardini et al., 1997], and the presence of viral genotype other than 1 [Martinot Peignoux et al., 1995; Shiratori et al., 1997]. Individual pretreatment features however have only limited predictive value and, thus, combinations of these factors might prove more useful [Conjeevaram et al., 1995]. Nonetheless, there are still patients who respond to IFN therapy but do not meet the specified pretreatment characteristics. Thus, it is difficult to use prognostic indicators to withhold therapy and it may not be ethical to withhold treatment from patients with a chronic viral illness that is progressive and potentially fatal. In this study, neither absolute E1 or E2 antibody titers nor anti-E1/HCV RNA or anti-E2/HCV RNA ratios allowed us to accurately discriminate nonresponders from sustained responders before the start of therapy. The sensitivity of the anti-E1/HCV RNA or anti-E2/HCV RNA ratio as a prognostic feature was equal to that of the HCV RNA titer on its own. Using each of these three markers separately, 23% of sustained responders would not have been treated. Thus, the best strategy still seems to start a therapy and monitor individual treatment outcome.

The early identification of non-responder patients during therapy would put on halt on ineffective treatment before completion. This would avoid substantial medical costs and needless patient suffering. This approach may become even more important in the near future because recommendations for IFN treatment in chronic hepatitis C patients will be for 12 to 24 months [Davis and Lau, 1997].

E2 antibodies have been lost in response to IFN therapy and lower or absent in sustained responders [Yokosuka et al., 1993; Saracco et al., 1994]. In agreement, we found a reduction of E2 antibody levels after treatment and this reduction was most pronounced and occurred most frequently in the sustained responder patient group. Saracco et al. [1994] reported that during IFN treatment, E1 antibody titers increased in comparison with pretherapy levels. In that study, over 80% of biochemical IFN responders were reactive to E1 after therapy. Thus, the presence or the appearance of anti-E1 during the course of treatment could be a predictor of a favorable prognosis. This contrasts with our observation that E1 antibodies tend to decline rapidly during the follow-up of patients with a sustained viral response. An explanation for these conflicting data is missing but may relate to the quality of the capturing antigen used or to the definition of response (biochemical versus virological). We estimate that, the decline in both E1 and E2 antibodies may be useful for monitoring response to IFN therapy.

In the sustained responder patient group, the decline in E1 antibody titer is larger and occurs more frequently than the drop in E2 antibodies. A significant difference was observed in the present study in the E1 antibody reduction between sustained responders and relapsers at the end of a 6-month course of IFN therapy. Monitoring anti-envelope antibodies may allow the clinician to discriminate between sustained and transient responders to IFN that can not be achieved with HCV RNA assessment. In contrast to the PCR, these enzyme immunoassays are reproducible, inexpensive, and automatable. A major problem that still remains relates to the genotype-specificity of the E1 recognizing antibodies. Inclusion of multiple genotype-specific antigens may further improve the assay.

In conclusion, monitoring E1 and E2 antibodies during treatment may be helpful in making treatment decisions. Patients without viraemic or biochemical responses showing no or only minor reduction of anti-envelope titers after 3 to 6 months of therapy should not receive further treatment with IFN alone but could be considered for combination therapy of interferon and ribavirin or enrollment in investigational studies. In viraemic and/or biochemical responders, monitoring of E1 antibody concentrations at 3 to 6 months following the start of therapy may be helpful in making therapeutic decisions.

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